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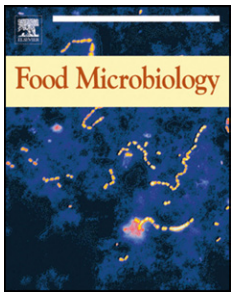

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Authors: Dimitra Dourou, Mohammed Salim Ammor, Panagiotis N. Skandamis, George-John E. Nychas

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Growth of *Salmonella* Enteritidis and *Salmonella* Typhimurium in the presence of quorum sensing signalling compounds produced by spoilage and pathogenic bacteria

Dimitra Dourou[1,2], D., Mohammed Salim Ammor[1,3], Panagiotis N. Skandamis[4], and George-John E. Nychas[1*]

[1]Laboratory of Microbiology and Biotechnology of Foods, [4]Laboratory of Food Quality Control and Hygiene, Department of Food Science and Technology, Agricultural University of Athens,

[2]Cranfield Health, Laboratory of Applied Microbiology, Cranfield University, Bedfordshire MK43 0AL, UK

[3]Total Quality Management Department, Centrale Laitière - DANONE, Twin Center, Tour A, 20100 Maârif, Casablanca, Morocco

Key words: *Salmonella enterica*; quorum sensing; acylated homoserine lactones; autoinducer-2; conductance.

Running title: *Salmonella* senses other quorated bacteria.

[*]author for correspondence: gjn@aua.gr

Abstract

The effect of acylated homoserine lactones (AHLs) and autoinducer-2 (AI-2) signalling compounds present in the cell-free culture supernatants (CFS), of *Pseudomonas aeruginosa*, *Yersinia enterocolitica*-like GTE 112, *Serratia proteamaculans* 00612, *Y. enterocolitica* CITY650 and *Y. enterocolitica* CITY844, on the growth of two *Salmonella* Enteritidis and two *S.* Typhimurium strains was assessed though monitoring of changes in conductance of the medium. Detection times (T_{det}), area and slope of conductance curves were recorded. Except for *P. aeruginosa* 108928, which was not found to produce AI-2, all other strains produced both AHLs and AI-2. Thereafter, aliquots (20% in the final volume) of these CFS were transferred into NZ Amine broth inoculated with *ca.* 10^3 CFU/ml of stationary phase cultures of each *Salmonella* strain. While the CFS of *P. aeruginosa* induced a shorter detection time, i.e. acceleration of the metabolic activity, the CFS of the other microorganisms increased the detection time of *Salmonella* strains compared to control samples (i.e. without CFS). Results indicate that the growth of *Salmonella* may be affected by the presence of Quorum sensing (QS) signalling compounds and/or other novel signals existing in CFS, produced by other bacterial species and confirm the complexity of bacterial communication.

1. Introduction

Salmonella enterica is one of the most prevalent pathogens associated with foodborne illness worldwide. It is second in the list of human zoonotic diseases across the EU (EFSA-ECDC, 2007), while in the USA, is a leading cause of foodborne illness resulting in an estimated 1.4 million infections, with more than 16,000 hospitalizations and nearly 600 deaths each year (Lynch et al., 2006). *Salmonella* infections can cause diarrhoea, fever, vomiting, and abdominal cramps. In the EU *Salmonella enterica* Typhimurium and *Salmonella enterica* Enteritidis are among the most frequently isolated serotypes (EFSA- ECDC, 2007). An array of physiological functions (e.g. colonization and virulence) that may contribute to its high prevalence have been reported to be regulated by systems of quorum sensing (QS; Walters and Sperandio, 2006; Kendall and Sperandio, 2007; Hughes and Sperandio, 2008).

QS involves the production of diffusible low-molecular-weight signalling molecules called autoinducers (AI), which have been referred to as bacterial pheromones (Stephens, 1986). When a critical level of such molecules is reached, signal-recognizing microorganisms sense that a sufficient level or “quorum” of bacteria is present and consequently regulate gene expression in favour of survival of the population (Federle and Bassler 2003; Smith et al., 2004). The role of cell-to-cell communication in food ecological niches has recently received attention from food microbiologists and a growing body of evidence suggests that bacterial food spoilage and poisoning could be regulated by QS (Smith et al., 2004; Ammor et al., 2008).

In many Gram-negative bacteria, quorum sensing is mediated by AHLs, generically called autoinducer-1 (AI-1), that are synthesized and recognized by QS circuits composed of LuxI and LuxR homologs (Miller and Bassler, 2001; Schauder and Bassler, 2001). *Salmonella* does not possess a *lux*I gene that codes for AHL

synthetase and thus does not produce AHLs. However, this organism does have a LuxR homolog, known as *Sdi*A, that enables detecting signals produced by other microbial species (Michael et al., 2001; Smith and Ahmer, 2003). In addition, *Salmonella* use two others QS systems, the *lux*S/AI-2 (Taga et al., 2001, 2003) and the AI-3/epinephrine/norepinephrine (Walters et al., 2006) to achieve intercellular signalling.

So far, the majority of studies on quorum sensing of *Salmonella* have focused on studying the genetic processes that regulate the synthesis, release and role of AI-2 production (Surette and Bassler, 1998; Surette et al., 1999; Taga et al., 2001, 2003). However, limited studies have focused on the ability of the pathogen to sense and respond to other bacterial species autoinducer signalling molecules (Michael et al., 2001; Smith and Ahmer, 2003). As foods and food processing environments harbour numerous types of microorganisms, some of which capable of producing QS signalling compounds, it is of primary importance to elucidate how interspecies communication modulates the growth responses (i.e. rate of metabolism and kinetic characteristics) of *Salmonella*.

Considering the above, the aim of this study was to investigate the effect of various QS signalling compounds e.g. AI-1 and/or AI-2 produced by *Y. enterocolitica*, *P. aeruginosa*, and *S. proteamaculans* (Pearson et al., 1994; Pearson et al., 1995; Throup et al., 1995; Gram et al., 1999; Winzer et al., 2002; Christensen et al., 2003; Bruhn et al., 2004; Atkinson et al., 2006; Van Houdt et al., 2007) on the kinetic characteristics of *S.* Enteritidis and *S.* Typhimurium. In all cases, *Salmonella* kinetic parameters were assessed by conductance measurements, a well-established methodology which allows monitoring of bacterial activity and kinetic characteristics (Richard et al. 1978; Firsteberg-Eden and Eden, 1984; Tranter et al., 1993; Silley and

Forsythe, 1996; Koutsoumanis et al. 1998; Chorianopoulos et al. 2006; Nychas et al. 2009, Chorianopoulos et al. 2010).

2. Materials and Methods

2.1. Bacterial strains and culture conditions

Pseudomonas aeruginosa 108928, *Y. enterocolitica*-like GTE112, *S. proteamaculans* 00612, *Y. enterocolitica* CITY650 and *Y. enterocolitica* CITY844 were propagated and subcultured in brain heart infusion (BHI; LAB M, Lancashire, UK) broth. Incubation at 28°C (*Y. enterocolitica*-like GTE112, *S. proteamaculans* 00612) or 37°C was allowed (*P. aeruginosa* 108928, *Y. enterocolitica* CITY650 and *Y. enterocolitica* CITY844) for 24 h prior to use. The identity of these strains was confirmed using sequence analysis of the V1-V3 region of the 16S rRNA (Rantsiou et al., 2006).

For the AHL bioassays, the AHL reporter strain *Agrobacterium tumefaciens* A136 (pCF218, pCF372) which detects most 3-oxo-N-acyl-homoserine lactones (Shaw et al., 1997) and the AHL producing strain *A. tumefaciens* KYC6 (pCF28; a 3-oxo-C8-HSL overproducer) (Fuqua and Winans, 1996), were resuscitated and subcultured in Luria Bertani (LB; Bertani, 1951) medium. This was supplemented with 4.5 μg/mL tetracycline and 50 μg/mL spectinomycin for *A. tumefaciens* A136 and icnucbated at 28°C for 28 h with agitation (160 rpm). *Hafnia alvei* 718 (a 3-oxo-C6-HSL producing strain) (Bruhn et al., 2004) was resuscitated and subcultured in BHI broth following incubation at 37°C for 24 h.

The reporter strain *Vibrio harveyi* BAA-1117^{TM} (BB170 *luxN*::Tn5, sensor 1^{-}, sensor 2^{+}), and the AI-2 producing strain *V. harveyi* BAA-1119^{TM} (BB152 *luxL*::Tn5,

100 autoinducer-1^{-}, autoinducer-2^{+}) were used for the AI-2 bioassays (Surette and Bassler,
101 1998); cultures were purchased from LGC Promochem (Teddington, Middlesex, UK).
102 These strains were grown in the autoinducer bioassay (AB) medium at 28°C for 24 h
103 with agitation (160 rpm). AB medium was prepared according to Greenberg et al.
104 (1979).

105 For conductance experiments, *S. enterica* serovar Enteritidis strains PT4 and PT7
106 (supplied by Division of Enteric Pathogens, Central Public Health Laboratory,
107 London, UK) and *S. enterica* serovar Typhimurium strains DT193 and DSM554 were
108 resuscitated and subcultured in tryptone soy broth (TSB; LAB M) at 37°C for 24 and
109 18 h, respectively.

110

111 *2.2. Cell-free culture supernatants (CFS) preparation*

112 *Y. enterocolitica*-like GTE112, *S. proteamaculans* 00612 *P. aeruginosa* 108928, and a
113 mixture of *Y. enterocolitica* CITY650 and *Y. enterocolitica* CITY844, herein after
114 called tester strains, were grown (*ca.* 10^{8}-10^{9} CFU/ml) in 30 ml of BHI broth under
115 conditions previously described. Cultures (25 ml) were then individually centrifuged
116 at 10,000 x g for 15 min (4°C) and the supernatants were filter-sterilized through a
117 0.22 µm pore-size cellulose acetate filter (Millipore, Malva SA, GR) to obtain CFS
118 from each tester strain. CFS was immediately screened for the presence of QS
119 signalling molecules as described below. To increase the range of existing QS
120 compounds in supernatants, in the case of *Y. enterocolitica* CITY650 and *Y.*
121 *enterocolitica* CITY844 a mixture (1:1, v/v) of both CFS was prepared (equal
122 populations) and utilized (unless otherwise stated) in all experiments of this study.
123 The remaining 5 ml from each culture were used to enumerate bacterial cells
124 population. In parallel the pH of the CFS was recorded (Metrohm 691 pH meter). The

initial pH of the CFS were; *Y. enterocolitica*-like GTE112 - pH 5.80; *P. aeruginosa* 108928 - pH 7.24; *S. proteamaculans* 00612 -pH 5.98; *Y. enterocolitica* CITY650 and CITY844, -pH 5.58

2.3. Screening for AHL signalling molecules

The *A. tumefaciens* A136 reporter strain was used for the screening of AI-1 like signalling molecules in CFS using a well diffusion assay (Ravn et al., 2001). Briefly, 1 ml of the culture was inoculated into 50 ml of melted ABT agar (1.5% agar; ABT per liter: 0.4 g $(NH_4)_2SO_4$, 0.6 g Na_2HPO_4, 0.3 g KH_2PO_4, 0.3 g NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.01 mM $FeCl_3$, 2.5 mg thiamine supplemented with 0.5% glucose and 0.5% casamino acids). This medium supplemented with the relevant antibiotics and 50 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; AppliChem GmbH, Darmstadt, Germany) and then immediately poured into 5.0 cm diameter Petri dishes. A portion (150 µl) of pure CFS or 20% CFS in NZ amine broth (NZA) was pipetted into wells (diameter 6.0 mm) punched in the solidified agar using a sterile Pasteur pipette. NZA contained per litre: 20 g NZ amine A (Sheffield Chemical Co. Norwich, NY) and 20 g proteose peptone No. 3 (Difco Laboratories, Detroit, MI). The plates were incubated at 28°C for 48 h. CFS of the AHL producing strain *A. tumefaciens* KYC6 and the reporter strain were used as positive and negative controls, respectively, in the above assay.

The development of blue colour (hydrolysis of X-Gal) in the plates indicated presence of AI-1 like substances. The area of the AHL induced zones surrounding the wells was measured using the Image J software (Wayne Rasband, NIH, Bethesda, Maryland, USA). The ratio of the coloured surface area in plates containing CFS to the coloured surface area of the AHL producing *A. tumefaciens* KYC6 was calculated

to allow for a semi-quantitative estimation of the AHLs produced by each tester strain. Two independent assays were performed with two replicate samples analysed in each assay.

2.4. Thin-layer chromatography (TLC)

Extracts for TLC were prepared from 5 ml cultures of the tester strains grown as exactly described above. Bacteria were removed by centrifugation and the supernatants were extracted twice with equal volumes of ethyl acetate acidified with 0.1% acetic acid (Shaw et al., 1997). The combined extracts were filtered (0.2 μm pore-size nylon filter; Whatman, Clifton, USA) and evaporated to dryness. Residues from the cultures were dissolved in 100 μl of HPLC-grade ethyl acetate. The AHLs compounds were determined using TLC plates (Nychas et al. 2009) and were visualised as blue spots on the TLC chromatographs. Images of the developed plates were obtained with a live view digital camera (Olympus, Live View Digital Camera, E-330, Olympus Imaging Corp., Tokyo, JP).

2.5. Screening for AI-2 signalling molecules

The *luxCDABE*-encoded luminescence response of the reporter strain *V. harveyi* BB170 was used as the basis for determining AI-2 activity in CFS of tester strains. The assay was performed as described by Lu et al. 2004, and Nychas et al. 2009

2.6. Monitoring the effect of the CFS on Salmonella *strains metabolic activity*

Growth of *Salmonella* strains was indirectly monitored by conductance measurements using the Malthus 2000 instrument (Radiometer International, Copenhagen, Denmark). A typical Malthus conductivity cell contains platinum

electrodes that allow the detection of conductance changes in liquid systems as a response to bacterial metabolism in the culture medium (Richard et al. 1978; Firsteberg-Eden and Eden 1984). Provided that the organisms are allowed to multiply, they will, in time, reach a number sufficient (10^5 to 10^7 CFU/ml) to cause a detectable conductance change. The conductance detection time (T_{det}, h) signal appears when three consecutive measurements exceed the minimum threshold of detection criteria (5 μS), and is defined as the time interval between the start of conductance monitoring and the beginning of the acceleration phase of the signal (Firstenberg-Eden and Eden, 1984; Silley and Forsythe, 1996). It is apparent that T_{det} depends on the microorganisms' population in the Malthus tubes (log CFU/ml), the growth kinetics of microorganism and the properties of the test medium, and can be considered as an indicator of increased microbial metabolism (Tranter et al., 1993; Silley and Forsythe, 1996; Koutsoumanis et al. 1998; Koutsoumanis and Nychas 2000). Conductance changes are expressed in microsiemens (μS) and are shown graphically as conductance curves. The greater the activity of the culture, the steeper is the slope or the higher is the area under the curve (Tassou and Nychas, 1994, 1995; Koutsoumanis et al., 1998, 2002; Koutsoumanis and Nychas, 2000; Skandamis et al., 2001; Giaouris et al., 2005; Chorianopoulos et al., 2008; .

2.7. Reaction cell preparation,

Conductance measurements were performed as follows: first aliquots of NZA broth (2.8 ml) were dispensed into sterile reaction Malthus tubes. Then aliquots (0.7 ml) of each tester strain CFS or BHI broth (0.7 ml; control) were then transferred to reaction cells to give final volumes of 3.5 ml. All reaction tubes (with or without CFS) were inoculated with each *Salmonella* strain to give an initial concentration of ca. 10^3

CFU/ml. The pH of broth (BHI) used was adjusted to the same value recorded on the corresponding CFS. Other non-inoculated with *Salmonella* tubes (3.5 ml) were used as an additional control. Malthus tubes were then incubated into the Malthus apparatus for 24 h at 37°C while the analyzer was adjusted to measure conductivity changes every 6 min. Tests with synthetic compounds was also performed (Chorianopoulos et al., 2010) and the baseline (measure in mS) of the instrument was constant and did not vary between different experiments

2.8 Experimental design and Data analysis

4 strains (2 S. Enteritidis and 2 S. Tymphimurium) x 4 CFS (extracted from *Y. enterocolitica*-like GTE112, *S. proteamaculans* 00612 *P. aeruginosa* 108928, *Y. enterocolitica* CITY650 and *Y. enterocolitica* CITY844) x 2 treatments (control cells with no addition of CFS and cells supplemented with CFS) x 12 reaction cells x 2 independent experiments. Thus, data from 768 conductance curves (i.e., changes of μS with time), derived from the above mentioned plan, were transferred to a Microsoft® Excel spreadsheet (Microsoft® Corp.) and were then fitted with the model of Baranyi and Roberts (1994). This primary model was used to empirically estimate the kinetic parameters of conductance changes: (i) the equivalent to lag phase period (LP; min), which in our case represents the time needed for conductance changes to occur in the growth medium and (*ii*) the Maximum Slope (i.e, the rate) of Conductance Changes (MSrCC, $\mu Smin^{-1}$; Richard et al., 1978; Nychas et al., 2009; Chorianopoulos et al., 2010), which represents the slope of the exponential phase of the sigmoial curve of changes in conductance due to microbial metabolism. For curve fitting, the in-house programme DMFit (Institute of Food Research, Norwich, United Kingdom) was used. The microbial activity of *Salmonella* was monitored indirectly by calculating the area

under the conductance/time curves using the trapezoidal rule (Lambert and Pearson, 2000; Chorianopoulos et al., 2006). The statistical significance ($p<0.05$) of the effect of each CFS on the activity of *Salmonella* strains, examined in two independent trials, was determined by the Student's *t* test with SPSS.

3. Results

3.1. Screening for QS signalling molecules

AHL production was screened using the *A. tumefaciens* A136 well diffusion assay (Figure 1) with results shown in Table 1. All testers strains produced AHLs, with *Y. enterocolitica*-like GTE 112 illustrating the highest production, followed by *S. proteamaculans* 00612, *Y. enterocolitica* CITY650 and CITY844, and *P. aeruginosa* 108928. The presence of at least one type of AHL signalling molecules in the CFS of each tester strain was additionally confirmed by TLC (Figure 2). As expected, the CFS of the reporter strain did not induce any colour development (results not shown).

AI-2-like activity in the CFS, assessed using the *V. harveyi* bioluminescence assay, revealed that with the exception of *P. aeruginosa* all strains were positive for the AI-2 extracellular signal. *Y. enterocolitica*-like GTE112 stimulated the highest light production in the reporter strain *V. harveyi* BB170 (18.7-fold, compared with the negative control) followed by *S. proteamaculans* 00612 (8.6-fold, compared with the negative control) (Table 2).

3.2. Salmonella kinetics with and without tester strains CFS

Salmonella strains were inoculated (*ca.* 10^3 CFU/ml) into NZA broth supplemented with 20% (v/v) of the tester strain CFS or 20% (v/v) of sterile BHI

249 broth (control). The pH of BHI was adjusted to the same value recorded on the
250 corresponding CFS with the purpose to rule out the influence of the pH on the
251 observed results. Conductance changes obtained by the Malthus instrument followed a
252 sigmoid curve typical of microbial growth, and thus, the model of Baranyi and
253 Roberts (1994) was used to empirically estimate the rate parameter MSrCC and the
254 lag phase period (LP) (Figure 3). In all studied cases, the model provided a good fit
255 (R^2>0.99).

256 The putative effect of signalling compounds present in CFS on T_{det}, on MSrCC, as
257 well as on the area of the conductance/time curves is shown in Tables 3-6. Non-
258 inoculated BHI broth samples supplemented or not with CFS did not exhibit any
259 change in conductance during incubation suggesting the absence of any metabolic
260 activity in the CFS (results not shown).

261 In comparison to control samples, tester strains grown in the presence of CFS,
262 containing autoinducer signalling compounds from *Y. enetrocolitica, P. aeruginosa*
263 and *S. proteamaculans,* proved to significantly influence ($p<0.05$) the kinetic
264 characteristics of all *Salmonella* strains, in most cases, but with different trends. In
265 particular the addition of CFS in the reaction cells influenced the kinetic parameter of
266 conductance area at the 100% of cases studied (i.e., 32 out of 32 cases). The detection
267 time (Tdec) was also influenced significantly with CFS, (in 31 out of 32 cases tested),
268 while the Maximum Slope of Conductance Changes (MSrCC) significantly differed
269 from that of the control samples in fewer cases (26 out of 32 cases; Table 3 - 6). It
270 needs to be mentioned that the CFS derived from *Y. enterocolitica* CITY650 and
271 CITY844 did not affect this kinetic characteristic of S. Enteritidis (i.e., both strains).
272 Specifically, the addition of CFS of *Y. enterocolitica*-like GTE112 or *S.*
273 *proteamaculans* 00612 into inoculated broth, reduced the MSrCC and increased the

T_{det} of *S.* Enteritidis PT4, *S.* Enteritidis PT7, *S.* Typhimurium DSM554 and *S.* Typhimurium DT193, indicating that CFS could cause suppression of the microbial metabolic activity (Tables 3 and 4). The area of the conductance/time curves of the tubes containing CFS was found to be significant lower that that of the control samples (32 out of 32 cases shown in Tables 3 to 6). Similar differences on the kinetic characteristics were observed when the mixed CFS of *Y. enterocolitica* CITY650 and CITY844 CFS was used with the exception of MSrCC of both *S.* Enteritidis strains that did not present any significant difference as compared to the control samples (Table 5). When the CFS of *P. aeruginosa* 108928 was used, however, the values of MSrCC and area of the conductance/time curves appeared to be significantly higher than in control samples for all *Salmonella* strains. In contrast, the T_{det} of *Salmonella* was shorter when the Malthus tubes were supplemented with CFS (Table 6).

4. Discussion

Most of the studies in the area of cell-to-cell communication have mainly focused on the molecular aspects of this phenomenon (e.g. how QS affects virulence, biofilm formation, sporulation or conjugation) and much less attention has been paid to the ecological context of how bacteria respond to both intra- and interspecies signals (Schauder and Bassler, 2001, Keller and Surette, 2006). This is of great importance since these signalling compounds are evident in food systems (Ammor et al., 2008; Nychas et al., 2009), but yet their specific role in such systems has not been fully elucidated. Indeed, the confirmation of presence ⁄ absence or determination of levels of QS compounds in foods does not answer the key questions as to how, for example, they influence other bacteria, what is their contribution (if any) on spoilage or how

298 food components are affecting the release and stability of QS molecules (e.g.,
299 inhibitors in the food matrix; Soni et al., 2008).

300 The presence of at least one type of AHLs and/or AI-2 signalling molecules in the
301 CFS, confirmed using different bacterial bioassays (Ravn et al., 2001; Surette and
302 Bassler, 1998) and/or TLC analysis (Shaw et al., 1997), suggests that these molecules
303 are likely associated with the altered metabolic activity of *Salmonella*, at least in the
304 tested broth and under the experimental conditions of this study. It is notable that the
305 response of *Salmonella* to the CFSs tested is quite diverse. In particular, no constant
306 inhibition or stimulating effect on growth of *Salmonella*, was observed. Instead, it
307 seems that the effect of AHLs or AI-2 signalling molecules on growth and metabolic
308 activity of the bacterium is rather dependent on the strains producing the signalling
309 compounds in the CFSs. However, although the observed differences between control
310 and treated cells were of low magnitude, they are statistically significant and basically
311 consistent over the independent trials, involving 768 independent samples in total.
312 These may suggest that in addition to the competitive (i.e., neutralization) effect of
313 QS inhibitors, which are potentially present in foods, on the activity of QS
314 compounds, the role of the latter on growth of pathogens is also affected by the type
315 of commensal food microflora capable of producing QS-like compounds.
316 Nonetheless, the existing reports on the role of QS-compounds in foods are
317 contradictory. For example, Soni et al. (2008) have reported that the presence of AI-2
318 molecules promoted the survival of *E. coli* O157:H7 cells, whereas the protective
319 effect of AI-2 molecules was negated in the presence of ground beef extracts that
320 contained significant amount of inhibitory activity. Although the contribution of
321 other unknown non-signalling compounds (e.g., products of proteolysis of
322 carbohydrate hydrolysis) also present in the CFS of the tester strains to the observed

phenomenon should not be ignored, an extensive GS-MC and HPLC analysis, of the tested reaction cells with or without CFS, in a similar study, did not reveal any difference in their composition (Chorianopoulos et al., 2010).

Data related to the rate of conductance changes and T_{det} have also been reported, when synthetic AHLs or cell-free culture fluids of microorganisms (i.e., spent medium) and cell-free meat extracts, containing AI-1 and AI-2 signals, were used to evaluate the synergistic or competitive effect of these signals on the evolution of other pathogenic or spoilage bacteria (Whan et al., 2003; Dunstall et al., 2005; Zhao et al., 2006; Nychas et al., 2009; Chorianopoulos et al., 2010). Contradictory results have been reported. For example, Dunstall et al. (2005) showed that N-benzoyloxycarbonyl-L-homoserine lactone (Z-HSL) and 3-oxyhexanoyl-DL-homoserine lactone (3-oxo-C6-HSL) significantly reduced the lag phase duration and increased the exponential growth rate of three strains of *P. fluorescens* isolated from raw milk, while in another study, Z-HSL was found to reduce both lag phase and exponential growth rate of two *P. fluorescens* strains isolated from pasteurized milk (Whan et al., 2003). In the present study, it was found that the use of synthetic compounds did not influence the detection time (Tdet) of *Salmonella* strains (results not shown), neither their kinetic parameters and this is in agreement with our previous studies involving the same pathogenic (Chorianopoulos et al., 2010) or spoilage (Nychas et al. 2009) bacteria. On the other hand, cell-free meat extract from spoiled meat containing QS compounds, increased the MSrCC of *P. fluorescens* but not of *S. marcenscens* (Nychas et al., 2009). Additionally, AI-2 signalling compounds present in the cell-free culture supernatant of *Escherichia coli* O157:H7, or in that of mixed enteric cultures, were shown to aid in the recovery and significantly enhance growth of stressed *E. coli* O157:H7 cells (Kolling and Matthews, 2007). Such variations

348 could possibly be attributed to the different sources e.g. producing bacteria of QS
349 signals and/or the different bacterial strains assayed.

350 The mechanism of action of QS signalling molecules produced by other bacteria on
351 *Salmonella* is not clearly elucidated. Knowledge of the exact chemical nature and
352 concentrations of auto-inducers present in the cell-free culture supernatants may aid in
353 elucidating their exact role on *Salmonella* growth and metabolic responses. However,
354 it could be hypothesized that *Salmonella* may have responded to the presence of
355 foreign AHLs and AI-2 compounds by utilising the signalling molecules to sense their
356 environment and regulate production of substances, (e.g. enzymes, metabolites etc.),
357 necessary for cell division and increase in population.

358 In addition to the role of QS signalling compounds in communicating cell density,
359 these compounds have also been suggested to act as proxies that provide individual
360 cells with information on the diffusion and flow properties of their environment
361 preventing the wasteful synthesis of “expensive” extracellular substances, such as
362 exoenzymes, bacteriocins, siderophores and other effectors (Redfield 2002; Keller and
363 Surette 2006; Hense *et al.* 2007). Provided that they remain in the cells immediate
364 environment, these metabolites, increase nutrient availability and ultimately benefit
365 the fitness of their producers (Redfield, 2002). This concept could possibly assist in
366 explaining the results of this study. Indeed, the addition in the reaction cells of the QS
367 signalling compounds and/or other novel signals existing in CFS, produced by the
368 tester strains, were rapidly mixed and diffused into the microenvironment of
369 pathogens, thereby altering *Salmonella* activity possibly through an over- or under-
370 production of necessary for growth substances (e.g. enzymes, metabolites etc;
371 Redfield, 2002).

372 The present findings suggest that: (i) the growth kinetic parameters as well as the
373 microbial activity of four *Salmonella* strains were affected by the addition of CFS
374 produced by other pathogenic and spoilage bacteria; and (ii) there was not a uniform
375 type of response in the bacterial strains tested. The response seems to be mostly
376 affected by the type of compound(s) present in each CFS and hence, the producer
377 strains. Direct extrapolation of such findings to real food ecosystems is currently
378 difficult; however, it is conceivable that our findings may represent situations of
379 interactions between bacteria and signalling compounds in the microenvironment of
380 foods. Further experimentation is required to elucidate the implications of such a
381 hypothesis. In addition, studies that will use microorganisms and their mutant strains
382 deficient in QS signalling production are required in order to identify the effect of
383 each type of signalling molecules on growth kinetics and more specifically on the
384 growth determinants of *Salmonella* (i.e. target genes and phenotypes). Such
385 approaches could potentially lead to the exploitation of these autoinducers as novel
386 antimicrobial agents and compounds to control microbial growth, survival and
387 virulence in foods.

388

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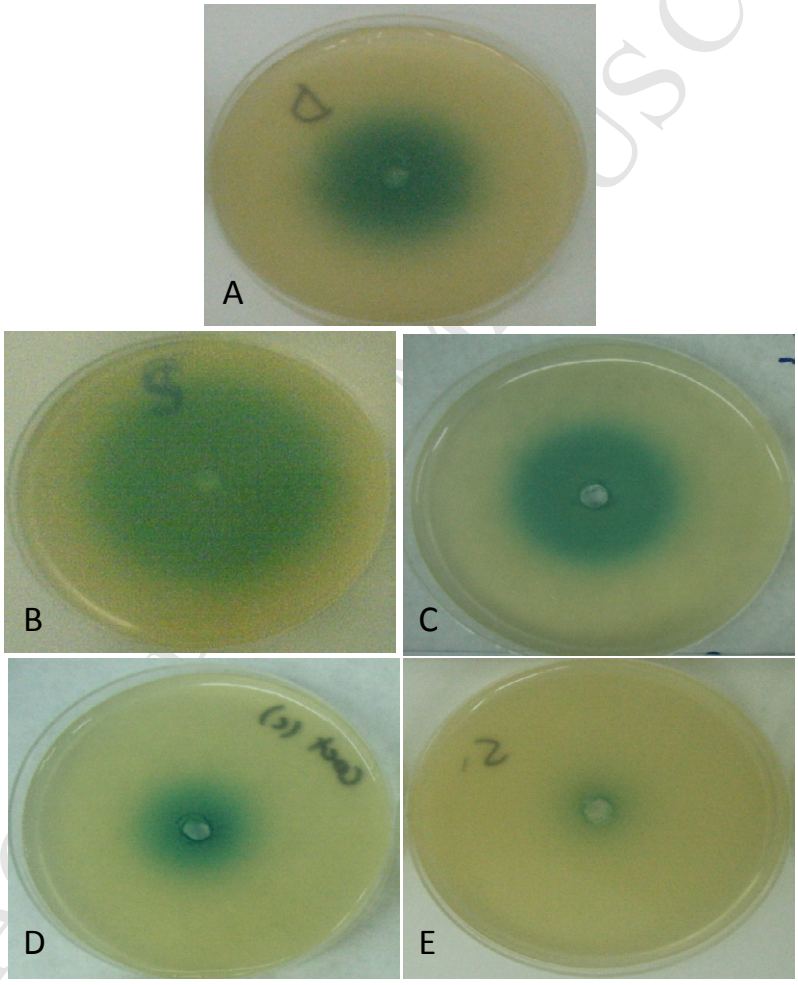
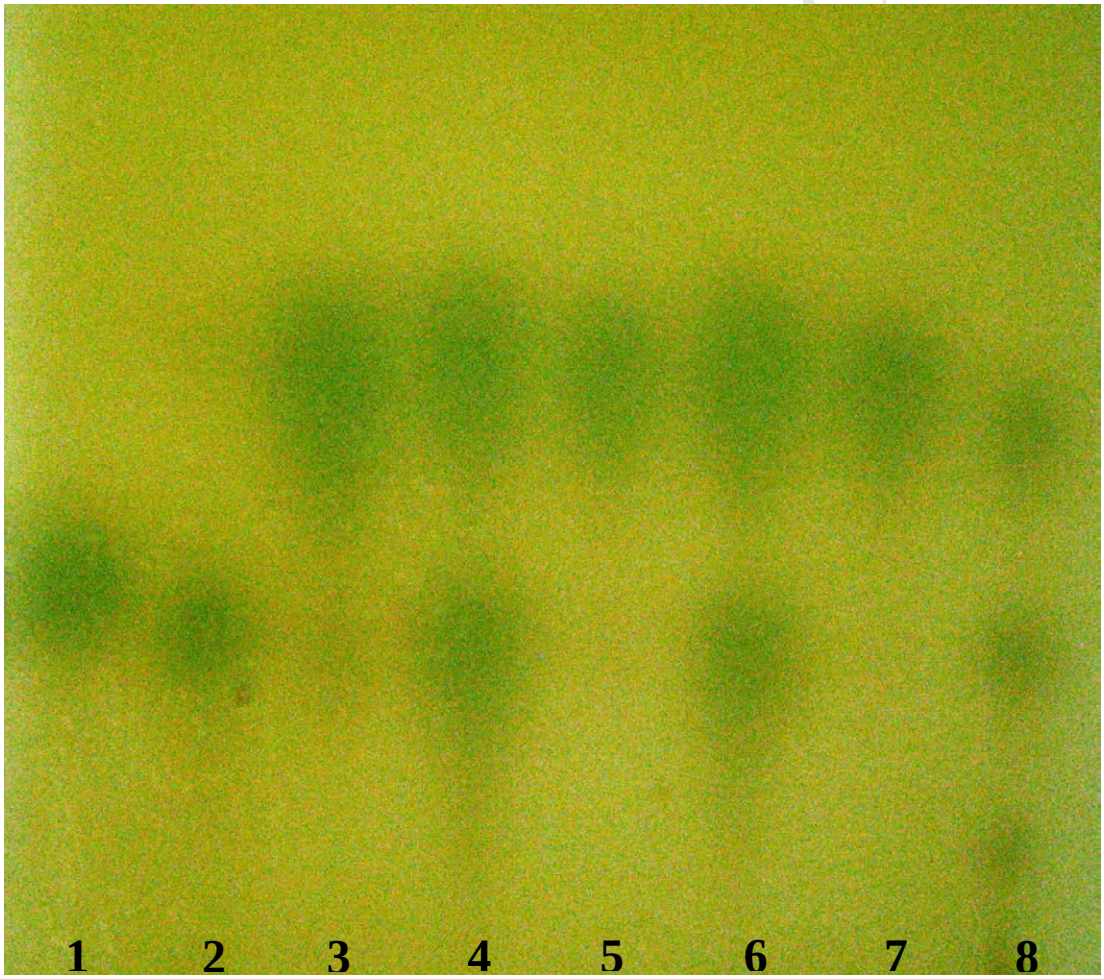
FIGURE 1. Representative photos exhibiting the presence of acylated homoserine lactones (AHLs) in the cell-free culture supernatants (CFS) of *A. tumefaciens* KYC6 (donor) (A), *Yersinia enterocolitica*-like GTE 112 (B), *S. proteamaculans* 00612 (C), *Y. enterocolitica* CITY650 and CITY844* (D) and *P. aeruginosa* 108928 (E). Tester strains CFS were added to wells in agar containing *A. tumefaciens* A136. Zones of AHL(s)-induced blue color production are seen surrounding the wells. * Mixture (1:1, v/v) of the CFS of the two *Y. enterocolitica* strains.



618 FIGURE 2. Thin layer chromatogram profiles of the AHLs present in the cell-free
619 culture supernatants (CFS) of the tester strains used in this study. Samples were
620 chromatographed on c18 reversed-phase TLC plates, developed with methanol/water
621 (60:40, v/v) and the spots were visualised by *A. tumefaciens* A136 reporter strain.
622 Lanes: (1) synthetic C6-AHL; (2) *A. tumefaciens* KYC6 (donor); (3) *H. alvei* 718; (4)
623 *Yersinia enterocolitica*-like GTE 112; (5) *S. proteamaculans* 00612; (6) *Y.*
624 *enterocolitica* CITY650; (7) *Y. enterocolitica* CITY844; (8) *P. aeruginosa* 108928.
626



627

TABLE 1. Autoinducer-1 activity of tester strains cell-free culture supernatant (CFS) in the well diffusion assay using the *A. tumefaciens* A136 reporter strain

		AI-1 activity	
Tester strain	Assay	CFS	20% CFS[a]
A. tumefaciens KYC6	1	1.00[b]	nt[c]
	2	1.00	nt
Y. enterocolitica- like GTE112	1	1.59	1.08
	2	1.54	1.18
P. aeruginosa 108928	1	0.47	0.18
	2	0.37	0.23
S. proteamaculans 00612	1	0.92	0.50
	2	0.98	0.47
Y. enterocolitica CITY650 and CITY844[d]	1	0.94	0.39
	2	nt	nt

[a] 20% CFS; the concentration of CFS used in conductance experiments.

[b] Results are displayed as the mean value (n=2) of the ratio of induction zone (area) of CFS of the tester strain to the induction zone (area) of the AHL producing *A. tumefaciens* KYC6.

[c] nt; not tested.

[d] Mixture (1:1, v/v) of the CFS of the two *Y. enterocolitica* strains.

649 TABLE 2. AI-2 activity in the cell-free culture supernatants (CFS) of *V. harveyi*

650 BB152 and the tester strains

Tester strain CFS	Relative AI-2 activity *
V. harveyi BB152	33.3
Y. enterocolitica- like GTE112	18.7
P. aeruginosa 108928	1.3
S. proteamaculans 00612	8.6
Y. enterocolitica CITY650	3.8
Y. enterocolitica CITY844	4.2

651 * Relative AI-2 activity (x-fold increase) was calculated as the ratio of luminescence of each CFS to the

652 control (negative) sample. Results represent mean values of two independent assays.

653

TABLE 3. Effect of addition of 20% in the final volume of cell-free culture supernatant (CFS) of *Y. enterocolitica*-like GTE112, in the growth medium, on the kinetics of *S.* Enteritidis (strains PT4, PT7) and *S.* Typhimurium (strains DSM554, DT193).

		Trial 1		Trial 2	
Microorganism	Kinetic parameters*	CFS	Control	CFS	Control
S. Enteritidis PT4	T_{det}	8.85** ± 0.37[a]	7.64 ± 0.46[b]	9.50 ± 0.34[a]	8.25 ± 0.14[b]
	MSrCC	2.10 ± 0.23[a]	2.02 ± 0.21[a]	2.13 ± 0.19[a]	2.69 ± 0.16[b]
	Area	13907 ± 3064[a]	21422 ± 3704[b]	14557 ± 2138[a]	30175 ± 642[b]
S. Enteritidis PT7	T_{det}	11.89 ± 0.48[a]	11.19 ± 0.18[b]	10.69 ± 0.72[a]	9.78 ± 0.32[b]
	MSrCC	1.23 ± 0.20[a]	1.50 ± 0.20[b]	1.72 ± 0.22[a]	2.09 ± 0.18[b]
	Area	7703 ± 1977[a]	13219 ± 3108[b]	10943 ± 2975[a]	23562 ± 3183b
S. Typhimurium DSM554	T_{det}	9.74 ± 0.23[a]	9.28 ± 0.04[b]	9.78 ± 0.34[a]	8.41 ± 0.32[b]
	MSrCC	2.21 ± 0.29[a]	2.45 ± 0.22[b]	2.35 ± 0.37[a]	2.84 ± 0.31[b]
	Area	13154 ± 3463[a]	23542 ± 2374[b]	15793 ± 2023[a]	28548 ± 2371[b]
S. Typhimurium DT193	T_{det}	9.43 ± 0.20[a]	8.77 ± 0.60[b]	8.21 ± 0.65[a]	7.63 ± 0.46[b]
	MSrCC	2.13 ± 0.31[a]	2.57 ± 0.53[b]	2.38 ± 0.19[a]	2.84 ± 0.47[b]
	Area	5462 ± 856[a]	7807 ± 2337[b]	5346 ± 911[a]	14909 ± 2637[b]

* T_{det}: Detection Time (h) as provided from Malthus instrument; MSrCC: Maximum Slope (rate) of Conductance Changes ($\mu Smin^{-1}$) as determined by fitting the conductance curves with DMFit software; Area: the area under the conductance/time curve.

** Mean values with different letters (a, b) within rows for each experiment are significant different ($p < 0.05$).

TABLE 4. Effect of addition of 20% in the final volume of cell-free culture supernatant (CFS) of *S. proteamaculans* 00612, in the growth medium, on the kinetics of *S.* Enteritidis (strains PT4, PT7) and *S.* Typhimurium (strains DSM554, DT193)

		Trial 1		Trial 2	
Microorganism	Kinetic parameters*	CFS	Control	CFS	Control
S. Enteritidis PT4	T_{det}	9.45** ± 0.27[a]	9.31 ± 0.40[a]	9.62 ± 0.39[a]	9.09 ± 0.39[b]
	MSrCC	1.68 ± 0.34[a]	1.97 ± 0.24[b]	1.74 ± 0.19[a]	2.00 ± 0.26[b]
	Area	27757 ± 3356[a]	31850 ± 2865[b]	11801 ±2657[a]	19796 ±3521[b]
S. Enteritidis PT7	T_{det}	13.20 ± 0.48[a]	12.34 ± 0.25[b]	12.47 ± 0.25[a]	11.08 ± 0.11[b]
	MSrCC	1.00 ± 0.25[a]	1.61 ± 0.17[b]	1.26 ± 0.30[a]	1.59 ± 0.14[b]
	Area	32482 ± 3123[a]	45869 ± 4650[b]	6501 ± 1505[a]	21014 ± 2602[b]
S. Typhimurium DSM554	T_{det}	9.89 ± 0.27[a]	9.62 ± 0.23[b]	10.01 ± 0.10[a]	9.53 ± 0.16[b]
	MSrCC	1.66 ± 0.19[a]	2.07 ± 0.23[b]	1.87 ± 0.36[a]	2.63 ± 0.25[b]
	Area	28925 ± 3401[a]	36807 ± 4863[b]	12554 ± 3045[a]	18004 ± 2109[b]
S. Typhimurium DT193	T_{det}	9.30 ± 0.24[a]	8.94± 0.40[b]	9.11 ± 0.16[a]	8.60 ± 0.35[b]
	MSrCC	1.83 ± 0.27[a]	2.61 ± 0.23[b]	1.76 ± 0.41[a]	2.73 ± 0.24[b]
	Area	27563 ± 2691[a]	31686 ± 2970[b]	9289 ± 3455[a]	16161 ± 3016[b]

* T_{det}: Detection Time (h) as provided from Malthus instrument; MSrCC: Maximum Slope (rate) of Conductance Changes ($\mu Smin^{-1}$) as determined by fitting the conductance curves with DMFit software; Area: the area under the conductance/time curve.

** Mean values with different letters (a, b) within rows for each experiment are significant different ($p < 0.05$).

TABLE 5. Effect of addition of 20% in the final volume of cell-free culture supernatant (CFS)[†] of *Y. enterocolitica* CITY650 and CITY844, in the growth medium, on the kinetics of *S.* Enteritidis (strains PT4, PT7) and *S.* Typhimurium (strains DSM554, DT193).

		Trial 1		Trial 2	
Microorganism	Kinetic parameters[*]	CFS	Control	CFS	Control
S. Enteritidis PT4	T_{det}	8.93[**] ± 0.63[a]	8.18 ± 0.68[b]	10.81 ± 0.14[a]	9.47 ± 0.09[b]
	MSrCC	1.83 ± 0.21[a]	1.56 ± 0.31[a]	2.05 ± 0.40[a]	2.22 ± 0.33[a]
	Area	14217 ± 1725[a]	19443 ± 6552[b]	15879 ± 2312[a]	25002 ± 4722[b]
S. Enteritidis PT7	T_{det}	11.33 ± 0.47[a]	10.93 ± 0.28[b]	12.13 ± 0.39[a]	11.55 ± 0.19[b]
	MSrCC	1.85 ± 0.14[a]	1.74 ± 0.23[a]	1.39 ± 0.14[a]	1.50 ± 0.30[a]
	Area	18260 ± 2609[a]	29931 ± 4602[b]	15000 ± 1520[a]	22263 ± 3056[b]
S. Typhimurium DSM554	T_{det}	9.57 ± 0.32[a]	9.11 ± 0.27[b]	10.22 ± 0.16[a]	9.34 ± 0.29[b]
	MSrCC	2.08 ± 0.15[a]	2.63 ± 0.20[b]	2.03 ± 0.30[a]	2.36 ± 0.30[b]
	Area	30889 ± 4441[a]	40207 ± 4432[b]	15917 ± 1912[a]	27289 ± 3674[b]
S. Typhimurium DT193	T_{det}	8.73 ± 0.53[a]	8.03 ± 0.45[b]	9.33 ± 0.14[a]	8.80 ± 0.14[b]
	MSrCC	1.92 ± 0.36[a]	2.35 ± 0.20[b]	1.59 ± 0.17[a]	1.75 ± 0.04[b]
	Area	14898 ± 1433[a]	19066 ± 3337[b]	13017 ± 1415[a]	17933 ± 1691[b]

[†] Mixture (1:1, v/v) of the CFS of the two *Y. enterocolitica* strains.

[*] T_{det}: Detection Time (h) as provided from Malthus instrument; MSrCC: Maximum Slope (rate) of Conductance Changes ($\mu S min^{-1}$) as determined by fitting the conductance curves with DMFit software; Area: the area under the conductance/time curve.

[**] Mean values with different letters (a, b) within rows for each experiment are significant different ($p < 0.05$).

TABLE 6. Effect of addition of 20% in the final volume of cell-free culture supernatant (CFS) of *P. aeruginosa* 108928, in the growth medium, on the kinetics of *S.* Enteritidis (strains PT4, PT7) and *S.* Typhimurium (strains DSM554, DT193)

		Trial 1		Trial 2	
Microorganism	Kinetic parameters*	CFS	Control	CFS	Control
S. Enteritidis PT4	T_{det}	7.07** ± 0.05[a]	7.30 ± 0.05[b]	6.34 ± 0.39[a]	6.88 ± 0.63[b]
	MSrCC	3.19 ± 0.23[a]	2.79 ± 0.361[b]	3.27 ± 0.36[a]	2.65 ± 0.41[b]
	Area	19532 ± 1991[a]	14579 ± 1240[b]	31578 ± 2285[a]	27279 ± 2674[b]
S. Enteritidis PT7	T_{det}	7.72 ± 0.08[a]	7.88 ± 0.16[b]	7.97 ± 0.67[a]	8.56 ± 0.24[b]
	MSrCC	2.28 ± 0.23[a]	1.89 ± 0.27[b]	2.51 ± 0.27[a]	2.22 ± 0.33[b]
	Area	13723 ± 2485[a]	10910 ± 1837[b]	30206 ± 4806[a]	27443 ± 1864[b]
S. Typhimurium DSM554	T_{det}	7.17 ± 0.08[a]	7.40 ± 0.05[b]	7.16 ± 0.45[a]	7.53 ± 0.05[b]
	MSrCC	3.13 ± 0.43[a]	2.76 ± 0.54[a]	3.66 ± 0.38[a]	3.24 ± 0.32[b]
	Area	18603 ± 2446[a]	14606 ± 2842[b]	30645 ± 3727a	26762 ± 1841[b]
S. Typhimurium DT193	T_{det}	6.63 ± 0.05[a]	6.79 ± 0.07[b]	7.01 ± 0.39[a]	7.40 ± 0.17[b]
	MSrCC	3.47 ± 0.23[a]	3.22 ± 0.12[b]	3.52 ± 0.41[a]	3.15 ± 0.29[b]
	Area	20369 ± 1461[a]	18418 ± 1037[b]	27014 ± 2771[a]	24180 ± 1500[b]

* T_{det}: Detection Time (h) as provided from Malthus instrument; MSrCC: Maximum Slope (rate) of Conductance Changes ($\mu S min^{-1}$) as determined by fitting the conductance curves with DMFit software; Area: the area under the conductance/time curve.

** Mean values with different letters (a, b) within rows for each experiment are significant different ($p < 0.05$).